Effects of ovarian reserve estimated with antral follicle counts on maturational ability and fertilizability of in vivo- and in vitro-grown oocytes in cattle

胞状卵胞数を指標とした牛卵巣予備能が

体内および体外発育卵子の核成熟能および受精能に与える影響

Katsuhisa NAGAI

Abbreviation

A I: anaphase I

AFC: antral follicular count

AMH: anti-müllerian hormone

ATP: adenosine triphosphate

BSA: bovine serum albumin

COCs: cumulus-oocyte complexes

DCHFDA: 2',7'-dichlorodihydrofluorescein diacetate

DPBS(-): Dulbecco's phosphate buffered saline without calcium and magnesium

ESH: oocytes with an enlarged sperm head with an anaphase I or telophase I chromosome

E_2: estradiorl-17β

FBS: fetal bovine serum

FSH: follicle-stimulating hormone

GV: germinal vesicle

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IBMX: 3-isobutyl-1-methylxanthine

IVC: *in vitro* culture

IVF: *in vitro* fertilization

IVG: *in vitro* growth culture

IVM: *in vitro* maturation

LH: luteinizing hormone

M I: metaphase I

M II: metaphase II

mBO: modified Brackett and Oliphant

OGCs: oocyte-granulosa complexes

OPU: ovum pick-up

OSF: oocyte-secreted factors

ROS: reactive oxygen species

T I: telophase I

TCM: tissue culture medium

2PN: oocytes with male and female pronuclei with a corresponding sperm tail

US: ultrasound

Contents

Preface · 1

Chapter I "***Fertilizability of oocytes derived from Holstein cows having different antral follicle counts in ovaries***"

Introduction · 3

Materials & Methods · 5

Results · 10

Discussion · 16

Summary · 19

Chapter II "***The relationship between AFC in a bovine ovary and maturational and developmental competences of in vitro-grown oocytes derived from early antral follicles***"

Introduction · 20

Materials & Methods · 22

Results · 28

Discussion · 35

Summary · 38

Summary and Conclusions · 39

Acknowledgements · 41

References · 42

Preface

The constant decline in fertility of dairy cattle has been a global problem for the last few decades. The conception rate of first insemination after parturition declined from 53.4% (1989) to 41.2% (2008) in Japan (Dochi et al., 2010). The non-return rate at 70 days after breeding declined from 54% (1996) to 45% (2007) in the United States (Norman et al., 2009). Many researchers have focused on nutrition, genetic improvement for milk production and endometritis as factors causing the decline of fertility (Grummer, 2007; Hansen, 2000; Sheldon et al., 2009), but the fertility of dairy cattle remains low. Fertility is a multi-factorial trait and its decline has been caused by a network of genetic, environmental and managerial factors. Their complex interactions make it difficult to determine the exact reasons for reduced fertility (Walsh et al., 2011).

Recently, size of ovarian reserve was proposed as a determining factor of fertility for mammalian female animals including humans (Broekmans et al., 2006) and cattle (Ireland et al., 2011). Major functions of ovaries are production of fertilizable oocytes having developmental competence that result in successful conception and secretion of sex steroid hormones that regulate the estrous cycle and sustain pregnancy. Ovarian reserve is defined as potential ability of these functions (te Velde and Pearson, 2002) and related to the number of primordial follicles (Wallace and Kelsey, 2010) in human. The number of primordial follicles is reported to be influenced by genetic and managerial factors, for example, heredity of early menopause (de Bruin et al., 2001), genes associated with premature ovarian failure in humans (Prakash et al., 2010), and maternal undernutrition during the first trimester of the gestation period in ewes (Rae et al., 2001) and cows (Mossa et al., 2013). Both the number of small antral follicles detected by ultrasonography (antral follicle count: AFC) in a pair of ovaries and the blood concentrations of anti-müllerian hormone (AMH) secreted from granulosa cells in growing follicles are reported

to correlate with the number of primordial follicles in human (Hansen et al., 2011) and in cattle (Ireland et al., 2008). Thus, AFCs and AMH levels have been used as indicators of ovarian reserve in human (Klinkert et al., 2005) and cattle (Burns et al., 2005). Counting antral follicles by ultrasonography is simple and noninvasive method, and enables repeated evaluation. In addition, there was no effect of stage of the estrous cycle on AFC in individual animals especially in cattle (Alvarez et al., 2000; Cushman et al., 2009).

It is reported that cows with low AFC, indicating small ovarian reserve, tend to have low fertility; for example, a long open period (Mossa et al., 2012), low steroidogenic capability (Jimenez-Krassel et al., 2009) and poor responsiveness to superstimulatory treatments (Ireland et al., 2007). In the case of humans, during the long lifetime of women, ovarian reserve decreases as aging. Therefore the relationship between oocyte quality and ovarian reserve in aged women is well investigated and the low quality of oocytes derived from aged women with small ovarian reserve was reported (Eichenlaub-Ritter et al., 2004; Oktay et al., 2015). In addition, the time of onset of menopause is closely correlated with ovarian reserve; small reserve resulted in earlier menopause (Depmann et al., 2015). The size of the ovarian reserve varies between individuals of the same species or strain; however, the relationship between ovarian reserve and the quality of oocytes in cattle is still unclear.

In the present study, the author focused on the relationship between the size of ovarian reserve and the intrinsic competence of bovine oocytes. In chapter I, I investigated the fertilizability of *in vivo*-grown oocytes derived from cows with different AFCs at recruitment and selection phase of follicular wave. In chapter II, I collected the oocytes from bovine ovaries with different AFCs and cultured for *in vitro* growth, focusing on the oocyte and follicular growth before recruitment phase of follicular wave. I also examined the mitochondrial activity, maturational and developmental competence of the oocytes.

Chapter 1

Fertilizability of oocytes derived from Holstein cows having different antral follicle counts in ovaries

Introduction

Cows with low AFC, estimated as low ovarian reserve, tend to have low fertility; for example, a long open period (Mossa et al., 2012), low steroidogenic capability (Jimenez-Krassel et al., 2009) and poor responsiveness to superstimulatory treatments (Ireland et al., 2007). However, the relationship between ovarian reserve and the quality of oocytes in cattle is still unclear, even though a reduced ovarian reserve has been linked to aging in humans associated with a reduced quality of oocytes (Eichenlaub-Ritter et al., 2004).

Ireland et al. (2007) reported that the developmental competences of bovine oocytes after *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) were similar regardless of AFC. In this study, oocytes were collected from slaughterhouse materials without considering the estrous cycle of animals, although it is well known that the estrous cycle and follicular size affect the quality of oocytes (Lonergan et al., 1994; Nagano et al., 2007). Therefore, oocytes should be collected at the same stage of the estrous cycle and follicular wave when evaluating oocyte quality. Ultrasound (US)-guided ovum pick-up (OPU) enables repeatable collections of oocytes from growing antral follicles because the ablation of follicles induces the recruitment of new follicular waves (Bergfelt et al., 1994). Therefore, if OPU is performed at the same interval, oocytes will be collected from follicles at the same status repeatedly. Silva-Santos et al. (2014) reported that OPU combined with the *in vitro* production of embryos resulted in a larger number

of transferable embryos per session from *Bos indicus* × *Bos taurus* hybrid females in a high-AFC group (≥ 40) than in a low-AFC group (≤ 10). However, the intervals between OPU sessions were random in that study. Hagemann et al. (1999) reported that oocytes with high developmental competence were collected from follicles at the recruitment phase (2 and 10 days after estrus) compared with those at the dominant phase (7 and 15 days after estrus) derived from ovaries of slaughtered dairy cows. However, AFCs in cows were not considered in that study.

There is also a possibility that the dynamics of follicular development differs between cows with different AFCs because an inverse correlation between AFC and the concentration of follicle-stimulating hormone (FSH) in serum has been reported (Burns et al., 2005; Ireland et al., 2007). Thus, it is also needed to examine follicular growth after OPU when considering the optimal interval of OPU for cows with different AFCs and its effect on the quality of oocytes.

In chapter I, to clarify the relationship between AFC and oocyte quality in cattle, the author collected COCs by US-guided OPU from cows in which the follicular wave was synchronized, and examined the morphologies of retrieved COCs. It is well known that blastocyst development is markedly affected by the number of oocytes cultured in group (Carolan et al., 1996; Ward et al., 2000). From the low-AFC cows, the number of oocytes collected is limited; therefore, the fertilizability of oocytes after IVM and IVF was evaluated. I also investigated the relationship between AFC and follicular growth after OPU.

Materials & Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee of Hokkaido University and Rakuno Gakuen University. The cows were kept at the experimental farms of Hokkaido University (n = 6; 3 lactating and 3 dry cows) and the Faculty of Veterinary Medicine of Rakuno Gakuen University (n = 8; all dry cows). Their age and parity at Hokkaido University were 7.5 ± 2.9 (mean ± standard deviation) and 4.3 ± 1.9, respectively. The age of the animals at Rakuno Gakuen University was 6.3 ± 1.2; however, their parity was unknown. US-guided OPU was carried out at 2 different intervals, namely, twice a week (3- or 4-day interval: short interval) and once a week (7-day interval: long interval) from July 2013 to June 2014. At Rakuno Gakuen University, 4 dry cows were used for short-interval OPU for 7 weeks from July to August (2, 4, 10 and 13 sessions), and 2 dry cows were used for long-interval OPU for 8 weeks from November to December (8 sessions each). Two dry cows were used for both long (8 sessions each) and short intervals (13 sessions each). At Hokkaido University, 2 dry cows were used for short-interval OPU for 5 weeks from January to February (9 sessions each), and 1 dry (7 sessions) and 3 lactating cows (5, 5 and 7 sessions) were used for long-interval OPU for 7 weeks from May and June. In total, 16 Holstein cows were used in this experiment.

Follicle aspiration system

A single-lumen needle (17 gauge, 490 mm long; Misawa Medical, Ibaraki, Japan) was connected to a 50-ml collection tube (Falcon 2070; Becton Dickinson, Franklin Lakes, NJ, USA) via a silicone tube (100 cm long, 1 mm internal diameter). The collection tube was warmed at 37˚C in a portable incubator (FV-5; Fujihira Industry, Tokyo, Japan) and the other silicone tube was connected to a vacuum pump with a foot-pedal switch (K-MAR-5000; Cook

Medical Technology, Brisbane, Australia). US-guided OPU was conducted using an ultrasound machine (HS-1500; Honda Electronics, Aichi, Japan) equipped with a 9.0-MHz long-handled micro-convex probe (HCV-3710MV; Honda Electronics), and the number of aspirated follicles was noted. Some OPU sessions were recorded using a digital video recorder connected to the US machine and the diameters of aspirated follicles were measured on a personal computer (56 out of 129 sessions). The diameters of follicles were calculated by halving the summed values for the follicular length of long and short axes. Antral follicles were divided into 3 categories according to their diameters (small: < 4 mm, intermediate: 4 - < 8 mm and large: ≥ 8 mm) because follicles of ≥ 4 mm in diameter are usually defined as representing the emergence of follicles (Ginther et al., 1989) and follicles of ≥ 8 mm in diameter start to express luteinizing hormone (LH) receptors (Bao et al., 1997).

Oocyte collection and classification

US-guided OPU was performed as previously described by Sasamoto et al. (2003; 2004). Before starting the experiments, follicle ablation under US guidance was carried out for synchronization of the emergence of the follicular wave (Bergfelt et al., 1994). Preceding follicle aspiration, cows were fixed in a treatment stall and injected with 2-4 ml of 2% lidocaine hydrochloride (Xylocine; AstraZeneca, Osaka, Japan) for epidural anesthesia. A long-handled micro-convex probe was inserted into the vagina after cleaning the vaginal region. The circuit from the tip of the aspiration needle to the collection tube was filled with flushing medium to avoid the attachment of contents within follicles to the inner surface and blood coagulation. Flushing medium consisted of Dulbecco's phosphate-buffered saline (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 1% calf serum (Invitrogen, Grand Island, NY, USA), 100 μg/ml streptomycin sulfate (Meiji Seika, Tokyo, Japan), 100 units/ml penicillin G potassium

(Meiji Seika Pharma, Tokyo, Japan) and 10 IU/ml heparin sodium (Ajinomoto Pharmaceuticals, Tokyo, Japan). All follicles detected by ultrasonography were counted and aspirated with 100 mmHg vacuum pressure (aspiration flow rate: 16.5 ml/min) as previously reported (Imai et al., 2006; Matoba et al., 2014). The recovered contents within follicles were poured through an EmCon filter (Immuno Systems, Spring Valley, WI, USA) and the filter was rinsed with about 200 ml of flushing medium without heparin. After rinsing, the contents of the filter cup were poured into plastic dishes (Falcon 351005) and cumulus-oocyte complexes (COCs) were detected under a stereomicroscope. Retrieved COCs were examined for their morphology under a stereomicroscope and divided into 4 grades: I) oocytes with several compact cumulus layers, II) oocytes denuded partially, III) oocytes denuded completely and IV) oocytes with expanded cumulus layers, as described previously (Merton et al., 2003).

In vitro maturation and fertilization

Unless stated otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). All of the collected COCs were matured individually as described previously (Nagano et al., 2013), then COCs from each animal were pooled and inseminated *in vitro* as described previously (Takahashi and Kanagawa, 1998). Briefly, each COC was cultured in a well individually in multi-well plates (Mini Trays 163118; NUNC, Roskilde, Denmark) filled with 6 ml of maturation medium in humidified atmosphere of 5% CO_2 in air at 39°C for 22 hr. Maturation medium consisted of TCM-199 (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen), 0.2 mM sodium pyruvate, 0.02 units/ml FSH (from porcine pituitary) and 1 μg/ml estradiol-17β (E_2). Then, all of matured COCs from each cow were pooled and inseminated in a droplet of IVF medium. Briefly, the COCs were co-cultured with spermatozoa (5×10^6 cells/ml) in 50-μl droplets (1-21 COCs/droplet) of fertilization medium covered with mineral oil in a

humidified atmosphere at 5% CO_2, 5% O_2 and 90% N_2 at 39°C for 18 hr. Fertilization medium was modified Brackett and Oliphant (mBO) isotonic medium (Brackett and Oliphant, 1975) supplemented with 2.5 mM theophylline and 3 mg/ml bovine serum albumin. After the thawing of frozen semen from a Holstein bull, motile sperm were separated using a Percoll (GE Healthcare, Pittsburgh, PA, USA) gradient (45% and 90%). After 18 hr, all oocytes were denuded by vortexing, fixed with fixative solution (75% ethanol and 25% acetic acid) on glass slides, and stained with 1% aceto-orcein solution, as described previously (Nagano et al., 2006). Fertilization status was examined under a phase contrast microscope as follows: oocytes with male and female pronuclei with a corresponding sperm tail (2PN), oocytes with an enlarged sperm head with an anaphase I or telophase I chromosome (ESH) and oocytes with more than two enlarged sperm heads or male pronuclei (polyspermy). We defined oocytes with 2PN as representing normal fertilization.

Experimental design

Antral follicular count was determined in all cows at each OPU session. Cows that showed AFC of ≥ 30 in at least one session during the experimental period were classified into the high-AFC group. The mean numbers of follicles in the high- and low-AFC groups for all experiments were 26.1 ± 6.1 (ranging from 12 to 50 follicles, n = 61) and 16.9 ± 5.4 (ranging from 7 to 26 follicles, n = 68) ($P < 0.05$), respectively. All follicles detected were subjected to aspiration; however, I sometimes failed to aspirate some of the small ones because of their positions. The ages of the animals in the high- and low-AFC groups were 6.9 ± 1.8 and 6.6 ± 2.6 years old, respectively. These cows were subjected to OPU at either a short (3 to 4 days) or a long (7 days) interval. The number of retrieved COCs was recorded, and the recovery rate of COCs based on aspirated follicles was calculated. After COC collection, the morphological

grade of the retrieved COCs was determined, and all of them were subjected to IVM and IVF. Then, the fertilization status of oocytes was examined.

Statistical analysis

Data obtained from dry and lactating cows from 3- and 4-day-interval OPU were combined because of their similarities. The numbers of collected COCs and the recovery rate of COCs in the high- and low-AFC groups were compared by two-way analysis of variance followed by Student's *t*-test. The data indicated by percentages were analyzed by chi-square test. Mean diameter of follicles was analyzed by Mann-Whitney's U test. All analyses were performed using software (JMP Pro 10.6, SAS Institute Inc., Cary, NC, USA). Values were considered significantly different at $P < 0.05$.

Results

There was a significant difference in the mean numbers of follicles between the high- and low-AFC groups at short and long intervals as shown in Table 1. There were interactions between AFC and OPU interval in the numbers of follicles aspirated and COCs collected ($P < 0.05$), but not in the recovery rate of COCs ($P = 0.28$). The recovery rates of COCs were similar between groups. The number of aspirated follicles was larger in the high-AFC group at each interval. At a long interval, the number of collected COCs was larger in the high-AFC group than in the low-AFC group. As shown in Table 2, when OPU was carried out at a short interval, there was no difference in the morphology of COCs between the high- and low-AFC groups. However, at a long interval, the percentage of grade I COCs in the high-AFC group was lower and the percentage of grade III COCs in the high-AFC group was higher than those in the low-AFC group, respectively ($P < 0.05$).

There were interactions between AFC and OPU interval in the mean number of aspirated follicles and the mean diameter of follicles ($P < 0.05$). As shown in Table 3, mean diameters of aspirated follicles were similar between the high- and low-AFC groups at each OPU interval. At a long interval, the proportion of large-sized follicles in the low-AFC group was greater than that in the high-AFC group ($P < 0.05$).

In the high-AFC group, there were no differences in follicular diameter and proportions of follicles in each diameter between the different OPU intervals. On the other hand, in the low-AFC group, the proportion of large-sized follicles at a long interval was higher and the proportion of small-sized follicles at a long interval was lower than those at a short interval ($P < 0.05$). The mean diameter of follicles was also larger at long interval than at short interval.

As shown in Table 4, at a short interval, total penetration rates were similar between the high- and low-AFC groups; however, the proportion of oocytes having 2PN in the high-AFC

group was higher than that in the low-AFC group ($P < 0.05$). At a long interval, both the proportions of oocytes having 2PN and the total penetration rate in the high-AFC group were lower than those in the low-AFC group ($P < 0.05$). In the low-AFC group, there was no difference in fertilization status between different OPU intervals; however, in the high-AFC group, 2PN and total penetration rates at a long interval were lower than those at a short interval ($P < 0.05$).

Table 1. Effects of OPU interval and antral follicle count (AFC) on the collection of cumulus-oocyte complexes (COCs)

OPU interval	AFC group	No. of cows (No. of sessions)	No. of follicles detected (n)	No. of follicles aspirated (n)	No. of COCs collected (n)	Recovery rate of COCs (%)
Short	High	3 (35)	24.5 ± 6.8 [a] (856)	22.6 ± 6.7 [a] (792)	6.2 ± 3.3 (217)	28.1 ± 16.6
	Low	5 (38)	17.4 ± 5.7 [b] (662)	16.0 ± 5.0 [b] (609)	4.7 ± 2.8 (178)	28.6 ± 15.3
Long	High	4 (26)	28.3 ± 4.3 [a]* (736)	27.1 ± 4.2 [a]* (705)	9.7 ± 3.6 [a]* (252)	35.7 ± 12.1
	Low	4 (30)	16.2 ± 4.8 [b] (486)	15.7 ± 4.7 [b] (472)	4.7 ± 3.4 [b] (142)	30.2 ± 19.4

Values are mean ± standard deviation.

[a, b] Different superscripts indicate a significant difference between AFC groups at the same OPU interval.

* Asterisks indicate a significant difference between the same AFC groups at different OPU intervals.

Table 2. Effects of OPU interval and antral follicle count (AFC) on the morphology of cumulus-oocyte complexes (COCs)

OPU interval	AFC group	No. of cows (No. of sessions)	No. of COCs collected	Proportion (n) of COCs graded as			
				I	II	III	IV
Short	High	3 (35)	217	43.8 (95)	25.8 (56)	27.6 (60)	2.8 (6)
	Low	5 (38)	178	47.1 (84)	23.6 (42)	27.0 (48)	2.2 (4)
Long	High	4 (26)	252	37.7 [b] (95)	20.2 (51)	36.5 [a]* (92)	5.6 (14)
	Low	4 (30)	142	47.9 [a] (68)	21.8 (31)	22.5 [b] (32)	7.7 (11)

[a, b] Different superscripts indicate a significant difference between AFC groups at the same OPU interval.

* Asterisks indicate a significant difference between the same AFC groups at different OPU intervals.

Table 3. Effects of OPU interval and antral follicle count (AFC) on aspirated follicular size

OPU interval	AFC group	No. of cows (sessions)	Mean no. of aspirated follicles (n)	Mean diameter (mm) of follicles (range)	Proportion (n) of follicles of each diameter		
					< 4 mm	4 - < 8 mm	≥ 8 mm
Short	High	3 (12)	23.1 ± 4.8 [a]* (277)	4.5 ± 2.2 (1.6 - 18.4)	54.2 (150)	38.6 (107)	7.2 (20)
	Low	3 (12)	16.7 ± 3.1 [b] (200)	4.3 ± 1.9 (1.6 - 11.6)	56.5 * (113)	39.0 (78)	4.5 (9)
Long	High	4 (16)	29.8 ± 5.0 [a]* (477)	4.7 ± 2.6 (1.8 - 21.4)	50.9 (243)	41.5 (198)	7.5 [b] (36)
	Low	4 (16)	14.4 ± 3.5 [b] (230)	5.2 ± 3.4 * (1.9 - 24.6)	46.5 (107)	41.3 (95)	12.2 [a]* (28)

Values are mean ± standard deviation.

[a, b] Different superscripts indicate a significant difference between AFC groups at the same OPU interval.

* Asterisks indicate a significant difference between the same AFC groups at different OPU intervals.

Table 4. Effects of OPU interval and antral follicle count (AFC) on fertilization statuses after *in vitro* maturation and fertilization

OPU interval	AFC group	No. of COCs (replicates)	Proportion (n) of oocytes with			Proportion (n) of oocytes penetrated by sperm
			2PN	ESH	polyspermy	
Short	High	217 (34)	29.0 [a]* (63)	13.8 (30)	8.3 (18)	51.2 * (111)
	Low	178 (35)	19.7 [b] (35)	14.6 (26)	10.1 (18)	44.4 (79)
Long	High	252 (26)	8.3 [b] (21)	8.7 (22)	4.8 (12)	21.8 [b] (55)
	Low	142 (29)	21.3 [a]* (30)	9.9 (14)	5.0 (7)	36.2 [a] (51)

2PN: male and female pronuclei, ESH: enlarged sperm head with anaphase I or telophase I oocyte.

[a, b] Different superscripts indicate a significant difference between AFC groups at the same OPU interval.

* Asterisks indicate a significant difference between the same AFC groups at different OPU intervals.

Discussion

In the present study, cows were allocated into high- and low-AFC groups based on the peak AFC (high: ≥ 30, low: < 30). The mean numbers of follicles in the high- (26.1 ± 6.1) and low-AFC groups (16.9 ± 5.4) in this study were similar to the criteria of bovine ovarian reserve (high: ≥ 25, low: ≤ 15 follicles) (Ireland et al., 2011). Most of the OPU sessions were carried out without corpus luteum because corpus luteum in each cow had regressed spontaneously until the second or third OPU session (at least 11 days after the first follicle ablation). Therefore, the timings of OPU performed in the present study correspond to 3.5 to 4.5 (short interval) and 7.5 days (long interval) after estrus because the emergence of a follicular wave occurs approximately 2 days after estrus (Sirois and Fortune, 1988) and 1.5 days after the ablation of follicles (Bergfelt et al., 1994).

At a short OPU interval, there were no differences in the distributions of COCs with different morphologies and follicles with different diameters between the high- and low-AFC groups. These results indicate that COCs were collected from follicles at the same phase in the follicular wave. After IVF, the total penetration rates in each group were also similar; however, the normal fertilization rate in the high-AFC group was higher than that in the low one. These results suggest that oocytes derived from cows with high AFC have higher competence for fertilization. In the present study, IVF media did not contain heparin, which induces semen capacitation (Parrish, 2014); thus, cumulus cells might play an important role in the present IVF system. A possible reason for the low fertilizability of oocytes collected at a long interval in the high-AFC group is the low quality of cumulus cells. Hyaluronan synthase 2 (HAS2), gremlin1 (GREM1) and pentraxin 3 (PTX3), genes expressed in cumulus (granulosa) cells are known to be indicators for evaluating the quality of human oocyte (Cillo et al., 2007) and especially HAS2 is also reported to be an indicator for predicting developmental competence of bovine

oocyte (Assidi et al., 2010; Salhab et al., 2010). Therefore, I should investigate the quality of cumulus (granulosa) cells derived from different AFC cows. In addition, Iwata et al. (2013) reported that oocytes from older beef cows had a lower mitochondrial DNA copy number than those from younger cows, resulting in lower rates of nuclear maturation, cleavage, and development to blastocyst *in vitro.* Ovarian reserve is considered to decrease according to maternal aging; therefore, the difference in AFC may also be considered to affect the number and activity of mitochondria. The ages of cows with different AFC used in the present study were similar; however, it is necessary to examine mitochondrial DNA copy number and mitochondrial activity of oocytes derived from different AFC cows in further study.

It is well known that extending the OPU interval decreases oocyte quality and developmental competence after IVF because of the higher incidence of atretic follicles (Hanenberg and van Wagtendonk-de Leeuw, 1997; Hagemann et al., 1999). It was reported that dominant follicles reach their maximum sizes at 6 to 7 days after estrus (Sirois and Fortune, 1988), and E_2 and inhibin secreted from these follicles suppress the development of other smaller follicles by the inhibition of FSH secretion (Ginther et al., 1996). These suggest the possibility that I collected COCs from follicles before follicular deviation at a short OPU interval, but from follicles after deviation at a long OPU interval. Indeed in the high-AFC group at long OPU interval, the proportion of follicles with different sizes was similar to that at short interval, and the percentages of COCs with low-graded morphology increased and high-graded morphology decreased. In addition, total penetration and normal fertilization rates of oocytes showed lowest values between groups. These results indicate that follicles in the high-AFC group start to degenerate until 7 days after OPU, and support the previous reports (Hanenberg and van Wagtendonk-de Leeuw, 1997; Hagemann et al., 1999). On the other hand, the mean diameter of follicles in the low-AFC group at long interval was larger than that at short interval

due to an increase in the proportion of large-sized follicles. In addition, normal fertilization and total penetration rates in the low-AFC group were similar between short and long OPU intervals. These results indicate that follicles in the low-AFC group continue to grow during extended period from 3-4 days to 7 days of OPU. The inverse correlation between AFC and the concentrations of FSH and inhibin in serum was reported in cattle (Burns et al., 2005); namely, low-AFC cows tended to show higher FSH and lower inhibin concentrations than high-AFC ones. Therefore, it is speculated that the deviation of growing follicles in low-AFC cows occurs later than 7 days after OPU probably due to high FSH concentration.

In conclusion, the oocytes derived from high-AFC cows have higher fertilizability than those from low-AFC cows when OPU is conducted at a 3- to 4-day interval. The difference of follicular growth between the high- and low-AFC cows observed in the present study indicates that the deviation of small follicles occurs earlier in the high-AFC group than in the low-AFC group. Therefore, the quality of oocytes derived from high-AFC cows may be impaired by extending the OPU interval to 7 days. Oocyte collection from cows by OPU at a short interval is recommended, especially for high-AFC cows. In further study, embryonic development after IVF should be examined by increasing the number of oocytes cultured in group and also embryo transfer should be performed in order to clarify the relationship between the developmental competence of oocytes to progeny and AFC.

Summary

In chapter I, to clarify the relationship between ovarian reserve and oocyte quality, cumulus-oocyte complexes (COCs) were collected repeatedly by ovum pick-up (OPU) from cows with high and low antral follicle counts (AFCs) at short (3-4 days) and long (7 days) intervals, and COC morphologies and oocyte fertilizability were examined. The relationship between AFC and follicular growth after OPU was also investigated. Cows showing AFC of ≥ 30 in at least one OPU session were grouped into the high-AFC group. At a short interval, follicular sizes and COC morphologies were similar between the different AFC groups. However, the normal fertilization rate was higher in the high-AFC group than in the low one, although total penetration rates were similar. At a long interval, the percentage of COCs with poor morphology in the high-AFC group was higher and the normal fertilization rate was lower than in the low one. In the low-AFC group, normal fertilization rates at short and long intervals were similar, and mean follicular size became larger at a long than at a short interval. However, mean follicular sizes at short- and long-interval OPU were similar in the high-AFC group. In conclusion, it is suggested that oocytes derived from cows with high AFC had higher fertilizability than those from cows with low AFC when OPUs were performed at a short (3-4 days) interval. However, oocyte quality in high-AFC cows was impaired by long-interval (7 days) OPU, possibly due to the deviation of follicular growth.

Chapter II

The relationship between AFC in a bovine ovary and maturational and developmental competences of in vitro-grown oocytes derived from early antral follicles

Introduction

The author investigated the relationship between ovarian reserve and oocyte quality of cows with high and low AFC, and showed that oocytes collected by OPU from the high-AFC cows had higher fertilizability than those from the low-AFC cows at 3 to 4 days OPU interval in chapter I. However, only in the high-AFC cows, the quality of oocytes was impaired by extending OPU interval to 7 days, possibly due to the earlier deviation of follicular growth compared to the low-AFC cows. From these results I have speculated that the growth rate of follicles is different between the high- and low-AFC cows, and the difference affects on the quality of oocytes. For eliminating the influence from estrous cycle and other developing or degenerating follicles on oocyte quality, I planned to investigate it using *in vitro* culture system of bovine oocytes.

For investigating developmental competence of bovine oocytes *in vitro*, oocytes derived from antral follicles of 2-8 mm in diameter are commonly used; however, these oocytes are already at various stage of follicular development or degeneration (Fuhrer et al., 1989; Nagano et al., 2007) and having various maturational and developmental competences depending on the stage (Lonergan et al., 1994; Fair, 2003; Nagano et al., 2006). Therefore, it is necessary to collect oocytes from follicles at early stage of follicular development. Recently, it was reported that *in vitro*-grown bovine oocytes derived from early antral follicles, just before

recruitment to follicular waves (0.5-1 mm in diameter), achieved good development to blastocysts corresponding to the development of *in vivo*-grown oocytes (Huang et al., 2013; 2014).

In chapter II, to investigate the relationship between ovarian reserve estimated with AFC and oocyte quality in cattle, I cultured oocyte-granulosa complexes (OGCs) collected from early antral follicles in bovine ovaries with high and low AFCs. During culture for *in vitro* growth (IVG), I examined viabilities of OGCs and the proliferation of granulosa cells. Nuclear maturation, mitochondrial activity and the accumulation of reactive oxygen species (ROS) in IVG oocytes were also evaluated. Moreover, the embryonic development of IVG oocytes derived from different AFC ovaries were investigated.

Materials & Methods

Collection of early antral follicles and in vitro growth culture of oocytes

Ovaries from Holstein cows obtained at a local abattoir were kept at 20°C and were transported to the laboratory within 6 to 10 hr after collection. After 3 washes in physiological saline, sliced ovarian cortex tissues (< 1 mm in thickness) were prepared using a surgical blade (No. 11) and stored in TCM-199 (Invitrogen) supplemented with 0.1% polyvinyl alcohol, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM sodium bicarbonate and 50 μg/ml gentamicin sulfate (isolation medium, pH 7.4, at 37°C), as described by Huang et al. (2013). Under a stereomicroscope, early antral follicles (0.5-1.0 mm in diameter) were dissected from the sliced ovarian tissues using a surgical blade (No. 20). OGCs were isolated from follicles using a pair of fine forceps and those with normal appearance were individually cultured in 96-well culture plates (Falcon 353872) with 200 μl of the growth medium for 12 days at 39°C in humidified air with 5% CO_2. Growth medium consisted of HEPES-buffered TCM-199 (Invitrogen) supplemented with 0.91 mM sodium pyruvate, 1 μg/ml E_2, 5% FBS (Invitrogen), 4 mM hypoxanthine, 4% polyvinylpyrrolidone (MW 360,000) and 50 μg/ml gentamicin sulfate. At the onset of IVG culture, OGCs were photographed under an inverted microscope (CK 40, Olympus, Tokyo, Japan) attached with a CCD camera (Moticam 2000, Shimadzu Rika Corporation, Tokyo, Japan), and viability of OGCs were determined by their morphologies as previously reported (Huang et al., 2013). During IVG culture, half (100 μl) of the growth medium was replaced with the same amount of fresh medium every 4 days.

Evaluation of granulosa cell proliferation during IVG culture

The OGCs with morphologically normal appearance at 8 and 12 days of IVG were used for granulosa cell count. The number of granulosa cells in a well was measured by a cell

counter (Luna FL Dual Fluorescence Cell Counter, Logos Biosystems, Virginia, USA). Briefly, after removing COCs and 175 µl of culture media from a well, 100 µl of Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS(-): Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.125% trypsin and 0.05% EDTA was added to a well. After 10 min of trypsinization and pipetting several times, 25 µl of FBS was added to stop the digestion. Then 18 µl of cell suspensions were mixed with 2 µl of acridine orange/propidium iodide cell viability kit (F23001, Logos Biosystems), and the numbers of live and dead granulosa cells were counted.

Pre-IVM and IVM of IVG oocytes

Before IVM (after pre-IVM) culture, the IVG oocytes recovered from morphologically normal OGCs were cultured individually in a well in microwell plates (Mini Trays 163118) that contained 6 ml of pre-IVM medium as described previously (Huang et al., 2014). Pre-IVM medium consisted of HEPES buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 2×10^{-6} units/ml FSH (from porcine pituitary), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µg/ml E_2, 10% FBS and 50 µg/ml gentamicin sulfate at 39°C in humidified air with 5% CO_2 for 10 hr. Some of IVG oocytes were subsequently cultured for another 22 hr at 39°C in humidified air with 5% CO_2 for IVM. Maturation medium consisted of HEPES buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/ml FSH, 1 µg/ml E_2, 10% FBS and 50 µg/ml gentamicin sulfate, and each IVG oocyte was cultured individually in a well in microwell plates (Nagano et al., 2013).

Evaluation of oocyte nuclear status before and after IVM

The IVG oocytes before and after IVM were denuded from cumulus cells by vortexing,

fixed with fixative solution (75% ethanol and 25% acetic acid), and stained with 1% aceto-orcein solution as described previously (Nagano et al., 2006). Nuclear status was examined under a phase-contrast microscope as follows: germinal vesicle (GV), metaphase I (M I), anaphase I/telophase I (A I/T I) and metaphase II (M II). Oocytes at M II stage were defined as matured.

Evaluation of mitochondrial activity and ROS accumulation in oocytes before and after IVM

Evaluation of mitochondrial activity and ROS accumulation in oocytes were performed as described previously (Huang et al., 2014). For evaluation of mitochondrial activity, IVG oocytes before and after IVM were treated with 500 IU/ml of hyaluronidase in DPBS(-) for 10 min, then oocytes were denuded from cumulus cells by repeat pipetting using a fine pipette. Denuded oocytes were incubated for 15 min in the dark at 37°C in DPBS(-) supplemented with 1 μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, Mitochondrial Membrane Potential Detection Kit JC100, Cell Technology Inc., Fremont, CA, USA), 1 μg/ml Hoechst 33342 and 10% calf serum. Images of oocytes were acquired using a digital fluorescent microscope (BZ-9000, Keyence, Osaka, Japan) and the mean fluorescence intensity of the images was calculated using analysis software (BZ-H2A, Keyence). Nuclear statuses of oocytes was evaluated by blue fluorescence of Hoechst 33342, and oocytes having GV before IVM and oocytes progressed to M II stage after IVM were subjected to the evaluation of mitochondrial activity. Membrane potentials of mitochondria in oocytes were calculated as the ratio of florescent intensity of activated mitochondria, expressed as red fluorescence of JC-1 staining, to less activated mitochondria, expressed as green fluorescence of JC-1 staining ($\Delta\psi m$, red/green fluorescent intensity).

For evaluation of ROS accumulation, oocytes before and after IVM were denuded

from cumulus cells by vortexing. Denuded oocytes were incubated for 15 min in the dark at 37°C in DPBS(-) supplemented with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA), 1 μg/ml Hoechst 33342 and 10% calf serum. Oocytes at GV stage before IVM and at M II stage after IVM were subjected to the evaluation of ROS accumulation, and images of them were acquired using a digital fluorescent microscope. The mean fluorescent intensity of the images was calculated using analysis software, and ROS accumulation in oocytes was defined as the mean fluorescent intensity of oocytes.

Fertilization and subsequent culture of IVG oocytes

After IVM, some oocytes were co-cultured with spermatozoa (5×10^6 cells/ml) in 100-μl droplets (5-13 oocytes/droplet) of fertilization medium covered with mineral oil in a humidified atmosphere at 39°C with 5% CO_2, 5% O_2 and 90% N_2 for 18 hr as described in chapter I. Inseminated oocytes (presumptive zygotes) were subsequently cultured as previously described (Takahashi and Kanagawa, 1998). Briefly, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in culture medium. Cumulus-free zygotes were cultured in 30-μl droplets (5-26 zygotes/droplet) of culture medium covered with mineral oil in a humidified atmosphere at 39°C with 5% CO_2, 5% O_2 and 90% N_2 for 6 days. The culture medium consisted of modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, 7 nonessential amino acids for minimum essential medium, 10 mg/ml insulin, 5 mM glycine, 5 mM taurine, 1 mM glucose and 3 mg/ml fatty acid-free BSA. Cleavage and blastocyst rates were determined after 2 days (approximately 30 hr) and 6 days (approximately 150 hr) of *in vitro* culture (IVC), respectively. Total cell numbers in a blastocyst obtained after 6 days of IVC were counted using an air-drying method (Takahashi and First, 1992).

Experimental group

AFC in an ovary was determined by the number of antral follicles of ≥ 2 mm in diameter. Usually, AFC means the number of follicle in a pair of ovaries, but I could not collect the pair of ovaries from a cow at the abattoir. For adapting the criteria of AFC (high: ≥ 25, intermediate: 16-24, low: ≤ 15) described by Ireland et al. (2011), I compared the AFC in an ovary detected by ultrasonography to by the direct inspection as a preliminary. In the preliminary study, the number of follicles in an ovary detected by direct inspection (15.5 ± 2.4, n = 4) after ovariectomizing was approximately double compared to the number of follicles detected by ultrasonography (7.0 ± 1.4, n = 4). Cushman et al. (2009) reported positive correlation between the number of antral follicles in one ovary and that in the contralateral ovary. Therefore, I allocated the ovary having more than 25 follicles of ≥ 2 mm in diameter as the high-AFC group and others as the low-AFC group. Then 189 and 342 ovaries were used as the high- and low-AFC groups, respectively.

Experimental design

During OGC collection, the number of early antral follicles and the number of collected OGCs in the high- and low-AFC groups were counted. OGCs having normal appearance were also counted and submitted to IVG culture. The viability of cultured OGCs was examined every 4 days. Some of OGCs were used for the evaluation of proliferation of granulosa cells, and nuclear maturation, mitochondrial activity and ROS accumulation of IVG oocytes.

Statistical analysis

The numbers of follicles and OGCs in high- and low-AFC ovaries were compared by Student's *t*-test. The viability of OGCs during IVG culture was compared by Tukey-Kramer's honestly significant difference. The number of granulosa cells at 8 and 12 days of IVG culture, mitochondrial activity and ROS accumulation in oocytes were compared by Student's *t*-test. The data of nuclear status was analyzed by chi-square test. Percentage data of cleavage and blastocyst rates were subjected to an arcsine square-root transformation, and analyzed by Student's *t*-test. Cell numbers in blastocysts were also analyzed by Student's *t*-test. All analyses were performed using software (JMP Pro 10.6). Values were considered significantly different at $P < 0.05$.

Results

As shown in Table 5, the mean number of early antral follicles dissected from one ovary in the high-AFC group was larger than that from the low-AFC groups ($P < 0.01$). The mean numbers of OGCs collected and OGCs having normal appearance in the high-AFC group were larger than those in the low-AFC group. However, the proportion of OGCs having normal appearance based on total OGCs was similar between the high- and low-AFC groups (66.0 ± 0.1% and 67.4 ± 0.2%, respectively).

As shown in Fig. 1, the proportions of live OGCs in both groups were similar and decreased until 8 days of IVG culture. As shown in Table 6, there was no difference in the mean number of granulosa cells between the high- and low-AFC groups at 8 days of IVG culture; however, the proportion of live cells in the high-AFC group was higher than that in the low-AFC group ($P < 0.05$). Numbers of granulosa cells in both groups increased from 8 to 12 days of IVG. Although the high-AFC group had larger number of cells than the low-AFC group at 12 days of IVG ($P < 0.05$), the proportions of live cells were similar between groups.

As shown in Table 7, the proportions of oocytes at GV stage before IVM culture were similar between the high- and low-AFC groups. However, the proportion of oocytes at M II stage after IVM culture in the high-AFC group was higher than that in the low-AFC group ($P < 0.05$).

As shown in Fig. 2, before IVM culture (at GV stage), the mitochondrial activity of oocytes in the high-AFC group was higher than that in the low-AFC group ($P < 0.05$). After IVM culture (at M II stage), the mitochondrial activities of oocytes in both groups increased compared to those before IVM ($P < 0.05$) and were similar between the high- and low-AFC groups. As shown in Fig. 3, before and after IVM culture, ROS accumulations of oocytes in both groups were similar; however, ROS accumulation decreased after IVM culture compared

to before IVM in both groups ($P < 0.05$).

The results of embryonic development of oocytes were described in Table 8. Cleavage rate in the high-AFC group tended to be higher than that in the low-AFC group ($P = 0.09$). There were no significant differences in both blastocyst rates based on inseminated and cleaved oocytes, although values of standard deviation were large. The numbers of cells in blastocysts in both groups were similar between groups.

Table 5. Relationship between AFC, numbers of early antral follicles and oocyte-granulosa complexes (OGCs)

AFC group	No. of ovaries (replicates)	AFC* (range)	No. of early antral follicles collected (n)	No. of OGCs collected (n)	No. of OGCs with normal appearance (n)
High (≥ 25)	189 (72)	37.4 ± 10.2 [a] (25 - 80)	19.5 ± 7.0 [a] (3687)	14.8 ± 5.6 [a] (2796)	10.0 ± 3.5 [a] (1806)
Low (< 25)	342 (69)	16.7 ± 4.7 [b] (9 - 24)	9.5 ± 3.9 [b] (3259)	6.9 ± 3.4 [b] (2372)	4.8 ± 1.5 [b] (1563)

Values are mean ± standard deviation.

* Antral follicle count: number of antral follicles of ≥ 2 mm in diameter detected by direct inspection.

[a, b] Different superscripts indicate a significant difference between groups ($P < 0.01$).

Table 6. Relationship between AFC and granulosa cell proliferation and survivability during IVG culture

IVG duration	AFC group	No. of OGCs used (replicates)	Mean number of granulosa cells ($\times 10^3$)		Proportion (%) of live cells
			total	live	
8	High	54 (5)	37.0 ± 22.9	33.1 ± 22.4	88.7 ± 15.4 [a]
	Low	45 (5)	32.5 ± 23.7	28.0 ± 23.7	79.9 ± 24.1 [b]
12	High	78 (9)	54.5 ± 32.1 [a]	39.3 ± 32.3 [a]	69.3 ± 28.8
	Low	72 (9)	37.9 ± 20.7 [b]	24.9 ± 16.0 [b]	71.1 ± 27.3

Values are mean ± standard deviation.

[a, b] Different superscripts indicate a significant difference ($P < 0.05$).

Table 7. Nuclear status of IVG oocytes derived from ovaries with high and low AFCs before and after IVM

IVM	AFC group	No. of cultured COCs (replicates)	Proportion (%) of oocytes at			
			GV	M I	A I / T I	M II
Before	High	70 (5)	90.0	8.6	2.3	0
	Low	65 (5)	83.1	17.0	0	0
After	High	157 (9)	0	15.9	1.9	82.1 [a]
	Low	118 (9)	0	22.9	7.6	69.5 [b]

GV: germinal vesicle, M I: metaphase I, A I/T I: anaphase I or telophase I, M II: metaphase II.

[a, b] Different superscripts indicate a significant difference ($P < 0.05$).

Table 8. Fertilizability and developmental competence of IVG oocytes derived from ovaries with high and low AFCs

AFC group	No. of oocytes (replicates)	Cleavage rate (n)	Blastocyst rate (%) based on		Cell number in blastocysts (n)
			inseminated oocytes	cleaved oocytes	
High	127 (9)	65.3 ± 11.7 (84)	11.4 ± 10.0	19.9 ± 20.3	81.7 ± 34.4 (13)
Low	80 (8)	45.0 ± 30.6 (42)	8.4 ± 15.9	13.7 ± 26. 1	86.6 ± 31.8 (5)

Values are mean ± standard deviation.

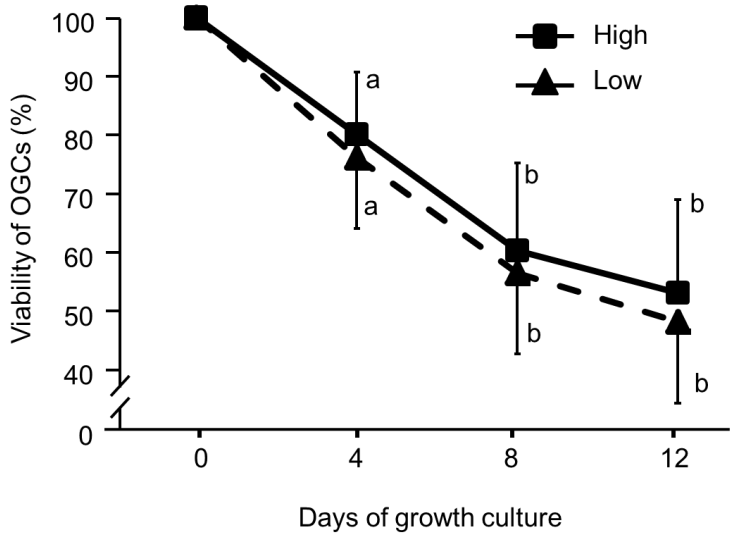

Fig. 1. Effects of antral follicle count (AFC) on viability of oocyte-granulosa complexes (OGCs) during *in vitro* growth culture

The numbers of cultured OGCs in the high- and low-AFC groups were 1,701 (68 replicates) and 1,490 (65 replicates), respectively. Solid line shows viability of OGCs in the high-AFC group and dashed line shows it in the-low AFC group.

[a, b] Different letters indicate a significant difference between days of growth culture in each group.

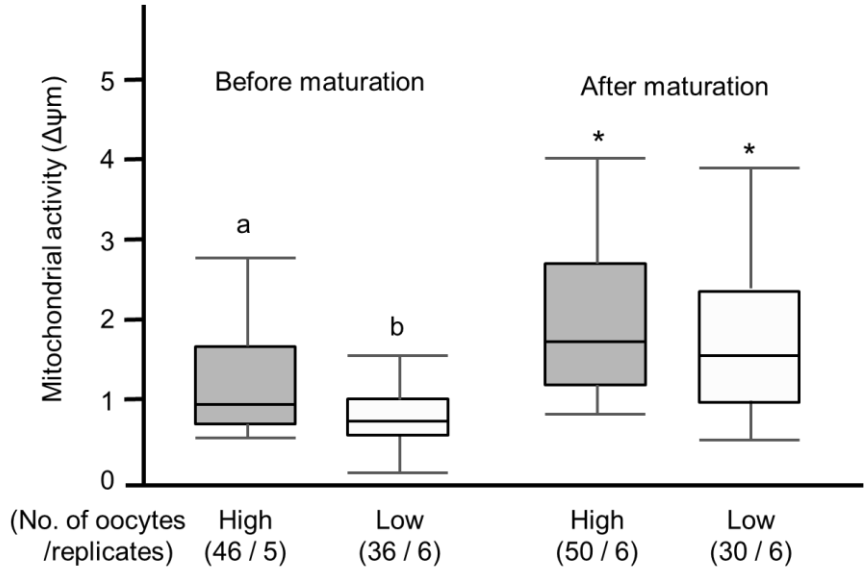

Fig. 2. Mitochondrial activity (Δψm) in oocytes before and after maturational culture in the high- and low-AFC groups evaluated by the ratio of red to green fluorescence intensity stained with JC-1

Oocytes at germinal vesicle stage and at metaphase II stage were used for evaluation of mitochondrial activity before and after *in vitro* maturation, respectively. Lines of the boxes delineate the 25th, 50th and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population.

[a, b] Different letters indicate a significant difference.

* Asterisk indicates a significant difference before and after maturation in same group.

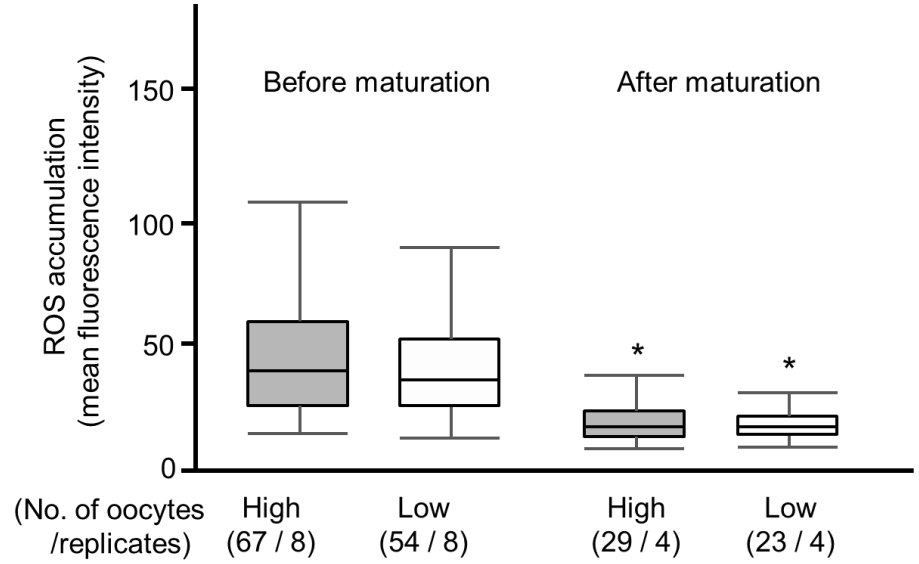

Fig. 3. Accumulation of reactive oxygen species (ROS) in ooplasm of oocytes before and after maturational culture in the high- and low-AFC groups evaluated by mean green fluorescence intensity stained with DCHFDA

Oocytes at germinal vesicle stage and those at metaphase II stage were used for evaluation of ROS accumulation before and after *in vitro* maturation, respectively. Lines of the boxes delineate the 25th, 50th and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population.

* Asterisk indicates a significant difference before and after maturation in same group.

Discussion

The high-AFC group had larger number of early antral follicles (≤ 1 mm) in an ovary than the low-AFC group, and the proportion of oocytes having normal appearance was similar between groups. These results correspond to the previous study reported that AFC in an ovary was positively correlated to number of follicles at various stages, and the proportion of healthy follicles was similar between the high- and low-AFC groups (Ireland et al., 2008). However, the number of granulosa cells at 12 days of IVG culture in the high-AFC group was larger than that in the low-AFC group. The high viability of granulosa cells at 8 days of IVG in the high-AFC group might contribute to larger number of granulosa cells at 12 days of IVG culture. These results seem to indicate that the function of granulosa cells is different depending on their origin of ovaries with different AFC. In the present study, oocytes in the high-AFC group also showed higher competence of nuclear maturation than that in the low-AFC group. Granulosa cells regulate the meiotic resumption of oocytes through gap junction (Sutton et al., 2003). It suggests that granulosa cells derived from the high-AFC cows are able to regulate the functions of meiosis more properly. Scheetz et al. (2012) reported that the cultured granulosa cells derived from the low-AFC cows showed lower secretion of estradiol, which is essential for granulosa cell proliferation (Gutiérrez et al., 1997). In the present study, I added the much concentration (1 μg/ml) of E_2 to IVG medium compared to that in the bovine dominant follicle of 10.5 mm in diameter (around 700 ng/ml) (Beg et al., 2001). It means that the E_2 is enough for proliferation of granulosa cells. However, Scheetz et al. (2012) also reported that the granulosa cells derived from the low-AFC cows secreted more progesterone than that from the high-AFC cows. For developing follicles healthily, the ratio of estradiol to progesterone is very important (Beg et al., 2001). In further study, I should examine the function of steroidogenesis of granulosa cells derived from different AFC cows. Another possibility of the cause of difference in the

proliferation of granulosa cells is the difference of oocyte function between groups. Differentiation and proliferation of granulosa cells are regulated by oocyte-secreted factors (OSFs) such as GDF-9 or BMP-15 (Gilchrist et al., 2008). Therefore, the secretion of OSFs from oocytes may be different between the high- and low-AFC groups. In the previous study, Scheetz et al. (2012) cultured only granulosa cells and examined the function of granulosa cells. For the correct evaluation of granulosa cell function of different AFC groups, the expression levels of OSFs in oocytes should be examined by co-culture with oocyte and granulosa cells.

Also the mitochondrial activity in oocytes before IVM was higher in the high-AFC group than in the low-AFC group. Mitochondrial activity is one of indicators of cytoplasmic maturation of oocytes (Benkhalifa et al., 2014). Huang et al. (2013, 2014) reported that IVG oocytes acquired high mitochondrial activity after 10-hr pre-IVM culture, and showed higher maturational and developmental competences compared to IVG oocytes without pre-IVM culture. Jeseta et al. (2014) reported that the kinetics of mitochondrial activity and adenosine triphosphate (ATP) production during IVM culture were influenced by the atretic grade of granulosa cells. This indicates the importance of the correlation between oocyte and granulosa cells. Low mitochondrial activity of IVG oocytes may be affected by lower number and also lower function of granulosa cells in the low-AFC group. In the ROS accumulation, there was no difference between the high- and low-AFC groups; nevertheless there was difference in the mitochondrial activity before IVM. ROS in ooplasm is considered to be a byproduct of electron transport chain activity in mitochondria when producing ATP (Richter, 1992). The results in the present study suggest that the activity of anti-oxidant enzymes in the high-AFC group is higher than that in the low-AFC group. Expression patterns of mRNA coding anti-oxidant enzymes were reported to change in according to days from follicular emergence (Valdez et al., 2005), follicular size and their status (Ceko et al., 2015) in cattle. It was also reported that mRNA

expression levels of glutathione peroxidase between dominant and subordinate follicles were different and that glutathione peroxidase could prevent cell apoptosis from oxidative stress during high steroidogenic period (Hayashi et al., 2010). Thus, evaluation of anti-oxidant enzyme expression in oocytes and granulosa cells derived from different AFC cows is necessary.

There was no obvious difference in blastocyst development after IVF between the high- and low-AFC groups; however, the cleavage rate tended to be high in the high-AFC group. The result corresponds to the high fertilizability of *in vivo*-grown oocytes in the high-AFC group in chapter I. There was no significant difference but the standard deviation of cleavage and blastocyst development in the low-AFC group was large, although the cell numbers in blastocysts was similar between groups. These results may indicate that some of oocytes derived from the low-AFC cows have relatively high developmental potential the same as those from the high-AFC cows. In the present study, I used only 5 to 26 IVG oocytes per replicate for evaluation of the embryonic development. It is well known that the number of oocytes cultured simultaneously affects the outcome of blastocyst production (Carolan et al., 1996; Ward et al., 2000). The low-AFC cows basically provide a low number of oocytes; therefore, the culture system for a few numbers of oocytes should be developed for evaluating the developmental competence of oocytes and for producing offspring effectively from cows with low AFC but having high potential as a domestic animal.

In conclusion, IVG oocytes derived from early antral follicles in ovaries with high AFC have higher maturational ability, and it is assumed to have higher fertilizability than those from ovaries with low AFC. Higher proliferation of granulosa cells during IVG culture and higher mitochondrial activity in ooplasm before IVM may support both nuclear and cytoplasmic maturation of oocytes from ovaries with high AFC.

Summary

To clarify the relationship between ovarian reserve and the developmental competence of bovine oocytes, oocyte-granulosa complexes (OGCs) collected from early antral follicles (≤ 1 mm) in ovaries with high (≥ 25) and low (< 25) antral follicle counts (AFCs) were used. OGCs derived from different AFC groups were cultured for *in vitro* growth (IVG) followed by *in vitro* maturation, fertilization and blastocyst formation. Viability of OGCs during IVG was similar between groups; however, OGCs in the high-AFC group had a larger number of granulosa cells than the low-AFC group at 12 days of IVG. After maturational culture, the proportion of matured oocytes in the high-AFC group was higher than that in the low-AFC group. Mitochondrial activity of oocytes before maturation in the high-AFC group was higher than that in the low-AFC group; however, the accumulation of reactive oxygen species was similar between groups. Cleavage rate in the high-AFC group tended to be higher than that in the low-AFC group, although blastocyst development was similar between groups. In conclusion, oocytes derived from ovaries with high AFC have higher maturational ability and fertilizability than those from low AFC. The difference may be caused by high proliferation of granulosa cells from ovaries with high AFC.

Summary and Conclusions

Recently, ovarian reserve was proposed as a factor of the fertility for mammalian females. Ovarian reserve is defined as potential ability of ovaries producing fertilizable oocytes having developmental competence that result in successive conception and secreting sex steroid hormones that induce the estrous cycle and sustain pregnancy. To estimate ovarian reserve of female animals, the number of small antral follicles in ovaries detected by ultrasonography (antral follicle count: AFC) is generally used, because it reflects the number of primordial follicles in a pair of ovaries in cattle. In addition, it is stable in individual animals at any day in an estrous cycle. It is also reported that cows with low AFC, estimating low ovarian reserve, tend to have low fertility; for example, a long open period, low steroidogenic capability and poor responsiveness to superstimulatory treatments. In the case of humans, low quality of oocytes derived from aged women with low ovarian reserve was reported. However, the relationship between the size of ovarian reserve and oocyte quality in cattle is no clear.

In chapter I, to clarify the relationship between ovarian reserve and oocyte quality, cumulus-oocyte complexes (COCs) were collected repeatedly by ovum pick-up (OPU) from cows with high and low AFCs at short (3-4 days) and long (7 days) intervals, and COC morphologies and oocyte fertilizability were examined. The relationship between AFC and follicular growth after OPU was also investigated. Cows showing AFC of $\geq$ 30 in at least one OPU session were grouped into the high-AFC group. At a short interval, follicular sizes and COC morphologies were similar between the different AFC groups. However, the normal fertilization rate was higher in the high-AFC group than in the low one, although total penetration rates were similar. At a long interval, the percentage of COCs with poor morphology in the high-AFC group was higher and the normal fertilization rate was lower than in the low one. In the low-AFC group, normal fertilization rates at short and long intervals were similar, and mean follicular size became larger at a long than at a short interval. However, mean follicular sizes at short- and long-interval OPU were similar in the high-AFC group. These

results indicate that oocytes derived from cows with high AFC have higher fertilizability than those from cows with low AFC when OPUs are performed at a short (3-4 days) interval. However, oocyte quality in high-AFC cows is impaired by long-interval (7 days) OPU, possibly due to the deviation of follicular growth.

In chapter II, to clarify the relationship between ovarian reserve and the developmental competence of bovine oocytes, oocyte-granulosa complexes (OGCs) collected from early antral follicles (≤ 1 mm) in ovaries with high (≥ 25) and low AFCs (< 25) were used. OGCs derived from different AFC groups were cultured for *in vitro* growth (IVG) followed by *in vitro* maturation, fertilization and blastocyst formation. Viability of OGCs during IVG was similar between groups; however, OGCs in the high-AFC group had a larger number of granulosa cells than the low-AFC group at 12 days of IVG. After maturational culture, the proportion of matured oocytes in the high-AFC group was higher than that in the low-AFC group. Mitochondrial activity of oocytes before maturation in the high-AFC group was higher than that in the low-AFC group; however, the accumulation of reactive oxygen species was similar between groups. Cleavage rate in the high-AFC group tended to be higher than that in the low-AFC group, although blastocyst development was similar between groups. These results suggest that oocytes derived from ovaries with high AFC have higher maturational ability and fertilizability than those from low AFC. The difference may be caused by high proliferation of granulosa cells from ovaries with high AFC.

In conclusion, oocytes derived from ovaries with high AFC have higher maturational competence and fertilizability than those from ovaries with low AFC, maybe due to the higher proliferation ability of granulosa cells in ovaries with high AFC. High proliferation ability of granulosa cells seems to contribute the early development of follicles in cows with high AFC than those with low AFC. In further study, the granulosa cell activity regulated by oocyte-secreted factors should be investigated.

Acknowledgements

The author would like to acknowledge Associate Professor Masashi NAGANO, Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, for valuable advice to this manuscript and review of the present manuscript.

The author greatly appreciate to Professor Yasuhiro KON, Laboratory of Anatomy, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, and Professor Toshio TSUBOTA, Laboratory of Wildlife Biology and Medicine, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, for reviewing of my manuscript, valuable advices and suggestions.

The author greatly thanks to Professor Seiji KATAGIRI, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, and Assistant Professor Yojiro YANAGAWA, Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, for providing encouragement and valuable advices. The author thanks to all colleagues in the Laboratory of Theriogenology, Graduate School of Veterinary Medicine, Hokkaido University, and members in the Laboratory of Theriogenology, Department of Veterinary Medicine, School of Veterinary Medicine, Rakuno Gakuen University.

I would like to express my sincere appreciation and thanks to Program for Leading Graduate Schools “Fostering Global Leader in Veterinary Science toward Contributing to One Health” and staffs supporting this program.

At last, I apologize to my parents for my deviation from ordinary carrier paths and appreciate for supporting my decisions.

References

Alvarez P, Spice, LJ, Chase Jr, CC, Payton ME, Hamilton TD, Stewart RE, Hammond AC, Olson TA, Wettemane RP. 2000. Ovarian and endocrine characteristics during the estrous cycle in Angus, Brahman and Senepol cows in a subtropical environment. *J. Anim. Sci*. **78,** 1291-1302.

Assidi M, Dieleman SJ, Sirard MA. 2010. Cumulus cells gene expression following the LH surge in bovine preovulatory follicles: Potential early markers of oocyte competence. *Reproduction* **140**, 835–852.

Baerwald AR, Adams GP, Pierson RA. 2003. Characterization of ovarian follicular waves in women. *Biol. Reprod*. **69**, 1023-1031.

Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE, Youngquist RS. 1997. Changes in messenger ribonucleic acid encoding luteinizing hormone receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. *Biol. Reprod.* **56**, 1158-1168.

Beg MA, Bergfelt DR, Kot K, Wiltbank MC, Ginther OJ. 2001. Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. *Biol. Reprod.* **64**, 432-441.

Benkhalifa M, Ferreira YJ, Chahine H, Louanjli N, Miron P, Merviel P, Copin H. 2014. Mitochondria: participation to infertility as source of energy and cause of senescence. *Int. J. Biochem. Cell Biol*. **55**, 60-64.

Bergfelt DR, Lightfoot KC, Adams GP. 1994. Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. *Theriogenology* **42**, 895-907.

Brackett BG, Oliphant G. 1975. Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reprod.* **12**, 260-274.

Broekmans FJ, Kwee J, Hendriks DJ, Mol BW, Lambalk CB. 2006. A systematic review of tests predicting ovarian reserve and IVF outcome. *Hum. Reprod. Update* **12**, 685-718.

Burns DS, Jimenez-Krassel, F, Ireland JLH, Knight PG, Ireland JJ. 2005. Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum FSH concentrations. *Biol. Reprod.* **73**, 54-62.

Carolan C, Lonergan P, Khatir H, Mermillod P. 1996. *In vitro* production of bovine embryos using individual oocytes. *Mol. Reprod. Dev.* **45**, 145-150.

Ceko MJ, Hummitzsch K, Hatzirodos N, Bonner WM, Aitken JB, Russell DL, Lane M, Rodgers RJ, Harris HH. 2015. X-Ray fluorescence imaging and other analyses identify selenium and GPX1 as important in female reproductive function. *Metallomics* **7**, 71-82.

Cillo F, Brevini TA, Antonini S, Paffoni A, Ragni G, Gandolfi F. 2007. Association between human oocyte developmental competence and expression levels of some cumulus genes. *Reproduction* **134**, 645-650.

Cushman RA, Allan MF, Kuehn LA, Snelling WM, Cupp AS, Freetly HC. 2009. Evaluation of antral follicle count and ovarian morphology in crossbred beef cows: investigation of influence of stage of the estrous cycle, age, and birth weight. *J. Anim. Sci.* **87**, 1971-1980.

de Bruin JP, Bovenhuis H, van Noord PAH, Pearson PL, van Arendonk JAM, te Velde ER, Kuurman WW, Dorland M. 2001. The role of genetic factors in age at natural menopause. *Hum. Reprod.* **16**, 2014-2018.

Depmann M, Faddy MJ, van der Schouw YT, Peeters PHM, Broer SL, Kelsey TW, Nelson SM Broekmans FJM. 2015. The relation between variation in size of the primordial follicle pool and age at natural menopause. *J. Clin. Endocrinol. Metab.* **100**, E845–E851.

Dochi O, Kabeya S, Koyama H. 2010. Factors affecting reproductive performance in high milk-producing Holstein Cows. *J. Reprod. Dev.* **56**, S61-S65.

Eichenlaub-Ritter U, Vogt E, Yin H, Gosden R. 2004. Spindles, mitochondria and redox potential in ageing oocytes. *Reprod. Biomed. Online* **8**, 45-58.

Fair T. 2003. Follicular oocyte growth and acquisition of developmental competence. *Anim. Reprod. Sci.* **78**, 203–216.

Fuhrer F, Mayr B, Schellander K, Kalat M, Schleger W. 1989. Maturation competence and chromatin behavior in growing and fully grown cattle oocytes. *J. Vet. Med.* **36**, 285-291.

Gilchrist RB, Lane M, Thompson JG. 2008. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum. Reprod. Update* **14,** 159-177.

Ginther OJ, Kastelic JP, Knopf L. 1989. Composition and characteristics of follicular waves during the bovine estrous cycle. *Anim. Reprod. Sci.* **20**, 187-200.

Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. 1996. Selection of the dominant follicle in cattle. *Biol. Reprod.* **55**, 1187-1194.

Grummer RR. 2007. Strategies to improve fertility of high yielding dairy farms: management of the dry period. *Theriogenology* **68**, S281-S288.

Gutiérrez CG, Campbell BK, Webb R. 1997. Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to

follicle-stimulating hormone, and morphological characteristics. *Biol. Reprod.* **56**, 608-616.

Hagemann LJ, Beaumont SE, Berg M, Donnison MJ, Ledgard A, Peterson AJ, Schurmann A, Tervit HR. 1999. Development during single IVP of bovine oocytes from dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. *Mol. Reprod. Dev.* **53**, 451-458.

Hanenberg EHAT, van Wagtendonk-de Leeuw AM. 1997. Comparison of 3, 4, or 7 day interval between oocyte collections for *in vitro* production results. *Theriogenology* **47**, 158.

Hansen LB. 2000. Consequences of selection for milk yield from a geneticist's viewpoint. *J. Dairy Sci.* **83**, 1145-1150.

Hansen KR, Hodnett GM, Knowlton N, Craig LB. 2011. Correlation of ovarian reserve tests with histologically determined primordial follicle number. *Fertil. Steril.* **95**, 170-175.

Hayashi KG, Ushizawa K, Hosoe M, Takahashi T. 2010. Differential genome-wide gene expression profiling of bovine largest and second-largest follicles: identification of genes associated with growth of dominant follicles. *Reprod. Biol. Endocrinol.* **8**, 11.

Huang W, Nagano M, Kang SS, Yanagawa Y, Takahashi Y. 2013. Effects of *in vitro* growth culture duration and prematuration culture on maturational and developmental competences of bovine oocytes derived from early antral follicles. *Theriogenology* **80**, 793-799.

Huang W, Kang SS, Nagai K, Yanagawa Y, Takahashi Y, Nagano M. 2014. Mitochondrial activity during pre-maturational culture in *in vitro*-grown bovine oocytes is

related to maturational and developmental competences. *Reprod. Fertil. Dev.* doi: 10.1071/RD14023.

Imai K, Tagawa M, Yoshioka H, Matoba S, Narita M, Inaba Y, Aikawa Y, Ohtake M, Kobayashi S. 2006. The efficiency of embryo production by ovum pick-up and *in vitro* fertilization in cattle. *J. Reprod. Dev.* **52**, S19–S29.

Ireland JJ, Ward F, Jimenez-Krassel F, Ireland JLH, Smith GW, Lonergan P, Evans ACO. 2007. Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. *Hum. Reprod.* **22**, 1687-1695.

Ireland JJ, Smith GW, Scheetz D, Jimenez-Krassel F, Folger JK, Ireland JLH, Mossa F, Lonergan P, Evans ACO. 2011. Does size matter in females? An overview of the impact of the high variation in the ovarian reserve on ovarian function and fertility, utility of anti-Müllerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. *Reprod. Fertil. Dev*. **23**, 1-14.

Ireland JLH, Scheetz D, Jimenez-Krassel F, Themmen APN, Ward F, Lonergan P, Smith GW, Perez GI, Evans ACO, Ireland JJ. 2008. Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. *Biol. Reprod*. **79**, 1219-1225.

Iwata H, Goto H, Tanaka H, Sakaguchi Y, Kimura K, Kuwayama T, Monji Y. 2013. Effect of maternal age on mitochondrial DNA copy number, ATP content and IVF outcome of bovine oocytes. *Reprod. Fertil. Dev*. **23**: 424-432.

Jeseta M, Ctvrtlikova KD, Hanzalova K, Hulinska P, Hanulakova S, Milakovic I, Nemcova L, Kanka J, Machatkova M. 2014. Mitochondrial patterns in bovine oocytes with

different meiotic competence related to their *in vitro* maturation. *Reprod. Dom. Anim.* **49,** 469-475.

Jimenez-Krassel F, Folger J, Ireland JLH, Smith GW, Hou X, Davis JS, Lonergan P, Evans ACO, Ireland JJ. 2009. Evidence that high variation in ovarian reserves of healthy young adults has a negative impact on the corpus luteum and endometrium during reproductive cycles of single-ovulating species. *Biol. Reprod.* **80**, 1272-1281.

Klinkert ER, Broekmans FJ, Looman CW, Habbema JD, te Velde ER. 2005. The antral follicle count is a better marker than basal follicle-stimulating hormone for the selection of older patients with acceptable pregnancy prospects after in vitro fertilization. *Fertil. Steril*. **83**, 811-814.

Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I. 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture *in vitro*. *Mol. Reprod. Dev.* **37**, 48-53.

Matoba S, Yoshioka H, Matsuda H, Sugimura S, Aikawa Y, Ohtake M, Hashiyada Y, Seta T, Nakagawa K, Lonergan P, Imai K. 2014. Optimizing production of *in vivo*-matured oocytes from superstimulated Holstein cows for *in vitro* production of embryos using X-sorted sperm. *J. Dairy Sci.* **97**, 743-753.

Merton JS, de Roos AP, Mullaart E, de Ruigh L, Kaal L, Vos PL, Dieleman SJ. 2003. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology* **59**, 651-674.

Mossa F, Walsh SW, Butler ST, Berry DP, Carter F, Lonergan P, Smith GW, Ireland JJ, Evans ACO. 2012. Low numbers of ovarian follicles >3 mm in diameter are associated with low fertility in dairy cows. *J. Dairy Sci*. **95**, 2355-2361.

Mossa F, Carter F, Walsh SW, Kenny DA, Smith GW, Ireland JLH, Hildebrandt TB, Lonergan P, Ireland JJ, Evans ACO. 2013. Maternal undernutrition in cows impairs ovarian and cardiovascular systems in their offspring. *Biol. Reprod.* **88**, 92, 1-9.

Nagano M, Katagiri S, Takahashi Y. 2006. Relationship between bovine oocyte morphology and *in vitro* developmental potential. *Zygote* **14**, 53-61.

Nagano M, Hishinuma M, Katagiri S, Takahashi Y. 2007. The relationship between oocyte morphology and ovarian status in cattle. *J. Reprod. Dev.* **53**, 953-958.

Nagano M, Kang SS, Koyama K, Huang W, Yanagawa Y, Takahashi Y. 2013. *In vitro* maturation system for individual culture of bovine oocytes using micro-volume multi-well plate. *Jpn. J. Ve.t Res.* **61**, 149-154.

Norman HD, Wright JR, Hubbard, SM, Miller, RH, Hutchison JL. 2009. Reproductive status of Holstein and Jersey cows in the United States. *J. Dairy Sci*. **92**, 3517-3528.

Oktay K, Turan V, Titus S, Stobezki R, Liu L. 2015. BRCA mutations, DNA repair deficiency, and ovarian aging. *Biol. Reprod.* **93**, 67, 1-10.

Parrish JJ. 2014. Bovine *in vitro* fertilization: *in vitro* oocyte maturation and sperm capacitation with heparin. *Theriogenology* **81**, 67-73.

Prakash GJ, Ravi Kanth VV, Shelling AN, Rozati R, Sujatha M. 2010. Mutational analysis of inhibin alpha gene revealed three novel variations in Indian women with premature ovarian failure. *Fertil. Steril.* **94,** 90-98.

Rae MT, Palassio S, Kyle CE, Brooks AN, Lea RG, Miller DW, Rhind SM. 2001. Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. *Reproduction* **122**, 915-922.

Richter C, 1992. Reactive oxygen and DNA damage in mitochondria. *Mutat. Res.* **275**, 249-255.

Salhab M, Papillier P, Perreau C, Guyader-Joly C, Dupont J, Mermillod P, Uzbekova S. 2010. Thymosins beta-4 and beta-10 are expressed in bovine ovarian follicles and upregulated in cumulus cells during meiotic maturation. *Reprod. Fertil. Dev.* **22,** 1206–1221.

Sasamoto Y, Sakaguchi M, Katagiri S, Yamada Y, Takahashi Y. 2003. The effects of twisting and type of aspiration needle on the efficiency of transvaginal ultrasound-guided ovum pick-up in cattle. *J. Vet. Med. Sci.* **65**, 1083-1086.

Sasamoto Y, Sakaguchi M, Nagano M, Katagiri S, Takahashi Y. 2004. Follicular development after ovum pick-up and fertilizability of retrieved oocytes in postpartum dairy cattle. *Jpn. J. Vet. Res*. **51**, 151-159.

Scheetz D, Folger JK, Smith GW, Ireland JJ. 2012. Granulosa cells are refractory to FSH action in individuals with a low antral follicle count. *Reprod. Fertil. Dev*. **24**, 327-336.

Sheldon IM, Price SB, Cronin J, Gilbert RO, Gadsby JE. 2009. Mechanisms of infertility associated with clinical and subclinical endometritis in high producing dairy cattle. *Reprod. Dom. Anim.* **44**, S1–S9.

Silva-Santos KC, Santos GMS, Koetz Jr, C, Morotti F, Siloto LS, Marcantonio TN, Urbano MR, Oliveira RL, Lima DCM, Seneda MM. 2014. Antral follicle populations and embryo production - *In Vitro* and *In Vivo* - of Bos indicus-taurus donors from weaning to yearling ages. *Reprod. Dom. Anim*. **49**, 228-232.

Sirois J, Fortune JE. 1988. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. *Biol. Reprod*. **39**, 308-317.

Sutton ML, Gilchrist RB, Thompson JG. 2003. Effects of *in-vivo* and *in-vitro* environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum. Reprod. Update* **9**, 35-48.

Takahashi Y, First NL. 1992. *In vitro* development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* **37**, 963-978.

Takahashi Y, Kanagawa H. 1998. Effect of oxygen concentration in the gas atmosphere during *in vitro* insemination of bovine oocytes on the subsequent embryonic development *in vitro. J. Vet. Med. Sci.* **60**, 365–367.

te Velde ER, Pearson PL. 2002. The variability of female reproductive ageing. *Hum. Reprod. Update* **8**, 141-154.

Valdez KE, Cuneo SP, Turzillo AM. 2005. Regulation of apoptosis in the atresia of dominant bovine follicles of the first follicular wave following ovulation. *Reproduction* **130**, 71-81.

Wallace WHB, Kelsey TW. 2010. Human ovarian reserve from conception to the menopause. *PLoS ONE* **5**, e8772.

Walsh SW, Williams EJ, Evans ACO. 2011. A review of the causes of poor fertility in high milk producing dairy cows. *Anim. Reprod. Sci*. **12**, 127-138.

Ward FA, Lonergan P, Enright BP, Boland MP. 2000. Factors affecting recovery and quality of oocytes for bovine embryo production *in vitro* using ovum pick-up technology. *Theriogenology* **54**, 433-446.